Baseline Susceptibility of Tobacco Budworm (Lepidoptera: Noctuidae) to Cry1F Toxin from *Bacillus thuringiensis*

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ABSTRACT Transgenic cotton, *Gossypium hirsutum* L., lines expressing both Cry1F and Cry1Ac insecticidal proteins from *Bacillus thuringiensis* (Bt) have been commercially available in the United States since 2005. Both Bt proteins are highly effective against tobacco budworm, *Heliothis virescens* (F.), and other lepidopteran pests of cotton. Although Cry1Ac has been available in Bt cotton since 1996, the Cry1F component is relatively new. As part of the proactive resistance management program for Cry1F/Cry1Ac cotton, a susceptibility-monitoring program is being implemented. Baseline variation in the susceptibility to Cry1F in field populations of tobacco budworm was measured. There was a three-fold variation in the amount of Cry1F needed to kill 50% of the neonates from 15 different field populations from the southern and central United States. Future variation in susceptibility of tobacco budworm populations to Cry1F or even resistance evolution could be documented based on this baseline data. A candidate diagnostic concentration was determined that may be efficiently used to identify individuals that potentially carry major alleles conferring field-relevant resistance to Cry1F before such alleles spread through field populations.

KEY WORDS *Heliothis virescens*, transgenic cotton, insecticide, resistance management

Transgenic varieties of cotton, *Gossypium hirsutum* L., expressing both the Cry1F and Cry1Ac insecticidal proteins from *Bacillus thuringiensis* have been commercially available in the United States since 2005. The Cry1Ac protein was derived from *B. thuringiensis* subsp. *kurstaki* and the Cry1F protein from *B. thuringiensis* subsp. *aizawai.* Cotton varieties developed by cross-breeding a line containing the Cry1F transformation event 281-24-236 with a line containing the Cry1Ac transformation event 3006-210-23 provide season-long broad-spectrum protection from feeding by lepidopteran pests (Haile et al. 2004, Langston et al. 2004, Leonard et al. 2005, Lorenz et al. 2005, Richardson et al. 2005, Smith et al. 2005, Willrich et al. 2005). One of the key economic pests targeted by Cry1F/Cry1Ac cotton is *Heliothis virescens* (F.) for which each Cry protein in this cotton individually shows high levels of activity against *H. virescens* (Blanco et al. 2003, Storer 2005), meeting the "high dose" criteria set out by the Environmental Protection Agency's Scientific Advisory Panel (USEPA 1998).

The Cry1F protein produced in event 281-24-236 is a synthetic protein consisting of the core toxin from Cry1Fa2 and parts of the C-terminal protoxin segments of the Cry1Ca3 and Cry1Ab1 proteins from *B. thuringiensis* [Cry1F(synpro); details provided in Gao et al. 2006]. In cotton plants, the full-length protein is cleaved to the insecticidal Cry1F toxin core by plant proteases (Gao et al. 2006). This protein shows biological activity against several cotton pests, including *H. virescens, Spodoptera exigua* (Hübner), *Spodoptera frugiperda* (J.E. Smith), *Helicoverpa zea* (Boddie), *Heliothis armigera* (Hübner), *Heliothis punctigera* (Wallengren), *Trichoplusia ni* (Hübner), and *Pseudoplusia includens* (Walker) (Luo et al. 1999, Iracheta et al. 2000, Liao et al. 2002, Gao et al. 2006). The mode of action of Cry1F is similar to that of the other Cry1 proteins, and it involves binding to receptors in the

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Table 1. Source description and initial no. of moths used for the establishment of *H. virescens* field-collected colonies in 2005

Location	County/parish	Host plant	Collection	Initial (P_0) moth no.	F_2 neonates yested
Georgia (GA1)	Coffee	Cotton	Aug.	240	640
Georgia (GA2)	Bacon	Cotton	Aug.	240	640
Georgia (GA3)	Taylor	Cotton	Aug.	120	640
Louisiana (LA1)	Franklin	Velvetleaf	July	60	256
Louisiana (LA2)	Franklin	Cotton	Aug.	180	512
Louisiana (LA3)	Franklin	Garbanzo	Sept.	120	512
Mississippi (MS1)	Washington	Velvetleaf	June	180	640
Mississippi (MS2)	Washington	Garbanzo	July	180	384
Mississippi (MS3)	Washington	Garbanzo	Aug.	180	512
N. Carolina (NC1)	Washington	Tobacco	Aug.	120	512
N. Carolina (NC2)	Wilson	Tobacco	Aug.	120	384
N. Carolina (NC3)	Johnson	Tobacco	Aug.	120	256
Texas (TX1)	Brazos	Garbanzo	July	180	384
Texas (TX2)	Brazos	Garbanzo	Aug.	180	384
Texas (TX3)	Brazos	Garbanzo	Sept.	120	384
USDA–ARS	Washington	Wild hosts	≥1971	Unknown	1,152

midgut cell membranes of susceptible insects followed by pore formation, disruption of the midgut epithelium, cessation of feeding, and death (Bravo et al. 2007). Specificity of activity is determined by the presence of specific receptors in the midgut of susceptible insects (Van Rie et al. 1989). Cry1F seems to bind to at least one Cry1Ac binding site in the midgut of *H. virescens.* This binding site has low affinity for Cry1F and additional receptors for Cry1F are thought to be present (Jurat-Fuentes and Adang 2001). The cadherin-like protein, a receptor for Cry1Ac that has been implicated in the resistance mechanism of the Cry1Ac-resistant YHD2 strain (Gahan et al. 2001), is not recognized by Cry1F (Jurat-Fuentes and Adang 2006).

There is a concern that the properties of Bt crops that make them effective can be at high risk of pest adaptation (Gould 1998) because season-long protein expression levels vary throughout the plant tissue and growing season. The resistance risk for pyramided insecticidal traits in crops (two or more proteins expressed that each exert a high level of control and with a low cross-resistance potential) is expected to be much lower than for a single-gene product (Roush 1998, Zhao et al. 2003). Although it is possible that low levels of cross-resistance could occur between Cry1Ac and Cry1F in *H. virescens* due to alterations in shared midgut receptors, it is unlikely that high levels of cross-resistance would occur. However, to mitigate the resistance risk, a resistance management program has been put in place for Cry1F/Cry1Ac cotton (USEPA 2005). A core component of that program is monitoring *H. virescens* populations for changes in susceptibility to the Bt proteins. There is considerable intraspecific variation in larval susceptibility to Bt proteins (Stone and Sims 1993, Luttrell et al. 1999, Blanco et al. 2004), so it is important to distinguish between shifts in susceptibility resulting from selection for resistance and natural variation in susceptibility. Although Cry1Ac has been used in Bt cotton since 1996, Cry1F is new to the system, and so a program was implemented in 2004 and 2005 to measure the baseline variation in susceptibility of *H. virescens* to Cry1F. Data from these bioassays were used to identify a candidate diagnostic concentration for screening potentially resistant tobacco budworms to be used in the ongoing monitoring program.

Materials and Methods

Populations. Fifteen tobacco budworm colonies were obtained from field-collected larvae (P_0) gathered from different crops at different times in regions of Alabama, Arkansas, Georgia, Louisiana, Mississippi, North Carolina, and Texas were Bt cotton adoption has reached ≥65% in recent years (Table 1). Insects were delivered overnight to the USDA Agricultural Research Service center in Stoneville, MS, where colonies were maintained and tested. Two- to 4 day-old F_1 moths (≤30 ±3 female:30 ± 3 male) were placed in carton buckets (3.7 liters, Neptune, Newark, NJ) and allowed to mate. Environmental conditions were maintained at 27 ± 2°C, 65 ± 10% RH, an d a photoperiod of 14:10 (L:D) h. Moths had free access to 10% sucrose solution. Males were removed after 2 d of moth enclosure to maximize genetic diversity. Male removal reduced the overrepresentation of certain males and enhanced the percentage of fertile females and the number of fertile eggs per female (Blanco et al. 2006). Females were left in the containers and eggs were harvested daily for three consecutive days. The *H. virescens* colony of the USDA–ARS center in Stoneville that has been maintained since 1971 was used for comparison.

Assays. F_2 neonates were tested for their response to a recombinant Cry1F (synpro) produced from *Pseudomonas fluorescens* (lot no. TSN 103748, Dow AgroSciences LLC, Indianapolis, IN), shown to be biologically equivalent to the plant-produced protein (Gao et al. 2006). Each colony was exposed to a series of eight (0, 1.5, 3.1, 5.2, 12.5, 25.0, 50.0, and 100.0 ng/cm^2) Cry1F dilutions applied to the surface of wheat germ diet. The initial stock solution was prepared in a buffer solution (pH 7.1) sonicated for 5 s in a ultrasonic processor to ensure complete dilution and adding 0.1% Triton X-100 to obtain a uniform spreading on the diet

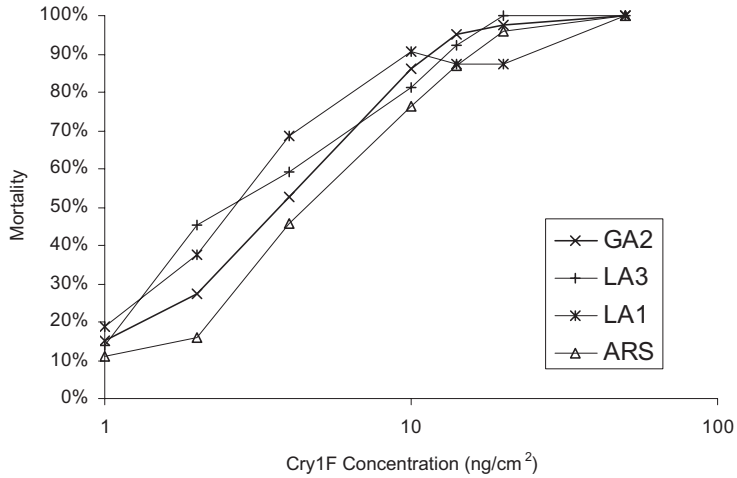

Fig. 1. Probit response of the *Heliothis virescens* reference colony (ARS) and of the three most Cry1F-susceptible strains.

surface (Siegfried et al. 2000). Each serial suspension prepared from the stock suspension was overlaid (21.5 μl/cm^2) on the surface of 16 wells of a 128-well tray (CD International, Pitman, NJ) and left to dry ($\approx$2.5 h). One $\leq$16 h neonate was placed in each cell then covered (BIO-CV-16, CD International). Bioassay trays with larvae were stored under the previously described environmental conditions. Each colony was tested two to five times by using F_2 larvae of the second or third oviposition days (Blanco et al. 2006). Bioassays were scored after 7 d considering as dead those larvae that did not move after probed and those that did not molt to second instar (growth inhibition concentration [EC], LC_{50}; Siegfried et al. 2000).

Data Analysis. Data (not corrected for mortality) were analyzed by probit analysis and the LC_{50} concentration (Siegfried et al. 2000) was calculated for each collection by using PROC PROBIT (SAS Institute 2001), which includes probit, logit, ordinal logistic, and extreme value (or gompit) regression models. By default, PROC PROBIT fits the probit (normal distribution) regression model. The choice of the distribution function F (normal for the probit model, logistic for the logit model, and extreme value or Gompertz for the gompit model) determines the type of analysis. For most problems, there is relatively little difference between the normal and logistic specifications of the model. Both distributions are symmetric about the value zero. The extreme value distribution (or Gompertz, used for colonies MS1 and NC1), however, is not symmetric, approaching zero on the left more slowly than it approaches one on the right. Differences in LC_{50} values of field-collected strains and the laboratory susceptible colony were considered significant if the 95% confidence limit (CL) of the resistance ratio at the LC_{50} level did not include 1.0 (Robertson and Priesler 1992).

Results and Discussion

There was approximately three-fold variation in Cry1F susceptibility data among 15 field-collected colonies. LC_{50} values ranged between 2.76 and 8.23 ng Cry1F/cm^2, whereas the value of the laboratory colony was established as 4.44 ng Cry1F/cm^2. Three colonies (Fig. 1, GA2, LA1, and LA3) were significantly more susceptible to this toxin, whereas seven (Fig. 2, MS1, MS2, MS3, NC1, NC2, TX2, and TX3) were significantly less susceptible to Cry1F (Table 2). The variability to this *B. thuringiensis* protein found in this study using a surface-treated technique is comparable to that previously found in tobacco budworm incorporating different *B. thuringiensis* toxins into insect artificial diet (8$\times$ [Stone and Sims 1993] and 5$\times$ [Luttrell et al. 1999]).

For pests against which transgenic crops provide very high efficacy, causing >99% mortality, only insects with very high levels of resistance would be able to survive on the transgenic crop and pass that resistance on to their offspring. Given that it has been shown that the level of Cry1F in cotton event 281-24-236 causes >99.9% mortality (Blanco et al. 2003, Storer 2005), identification of a laboratory concentration of Cry1F that reliably causes 99% mortality provides a candidate for such a discriminating concentration (Marçon et al. 2000). The maximum value for the concentration that killed 99% of the larvae (LC_{99}) of the field colonies was 186 ng Cry1F/cm^2 (TX2), the other values ranged from 32 (GA2) to 92 (TX3) ng Cry1F/cm^2. The LC_{99} for the susceptible colony was 46 ng Cry1F/cm^2. A proposed diagnostic concentration of 100 ng Cry1F/cm^2 based on pooled data from the 15 colonies (LC_{99} = 61.7 ng/cm^2, fiducial limits [FL] = 45.6–90.5, slope 1.405 [$\pm$0.04 SE]) and the fact that no survival or development beyond first instar was observed in this concentration in all tested colonies is highly conservative for Cry1F/Cry1Ac cotton. Additional tobacco budworm sensitivity data that can be

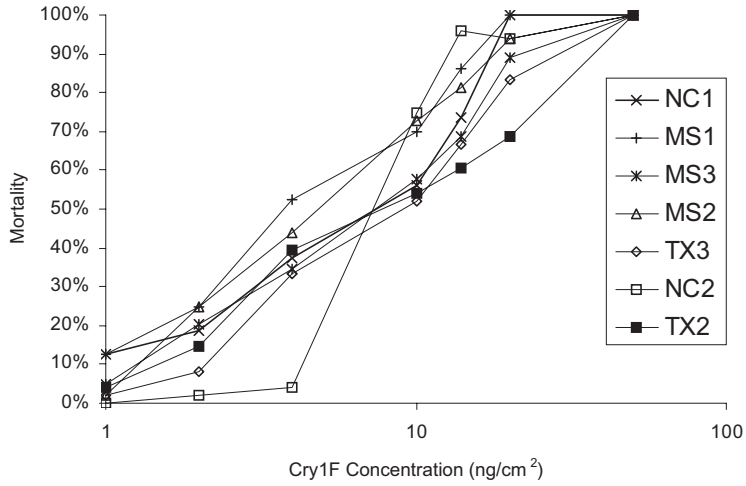

Fig. 2. Probit response of the least Cry1F-susceptible *Heliothis virescens* strains.

gathered during the early years of commercialization of Cry1F/Cry1Ac cotton can bolster this baseline and be used to validate the diagnostic concentration. Future variation in Cry1F susceptibility in field collections of tobacco budworm could be compared with the baseline variation established here to provide early warning of potential resistance.

Being a phenotypic assay, a diagnostic concentration would detect putative resistant homozygotes if resistance is recessive or near recessive, and it would detect putative heterozygotes if resistance is codominant or dominant. High levels of resistance to Bt crops are anticipated to be recessive traits (Ferre and Van Rie 2002). Although the F_2 screen may be effective at measuring the frequency of rare recessive resistance alleles (Andow and Alstad 1998), a phenotypic screen such as the diagnostic concentration bioassay, may be better suited for long-term routine monitoring for relevant increases in resistance frequency as part of a proactive resistance management program. Resistance management programs for Bt crops that are "high dose" against the target pests are based on an assumption that resistance is rare (in the order of 10^{-3}) and recessive. Detection of such alleles at a frequency in the order of 10^{-3} would not lead to an alteration of the resistance management strategy. For high dose crops with non-Bt refuges, the rate at which recessive resistance allele frequency may increase before identification in an effective monitoring program based on a phenotypic screen is limited (Gould 1998), and this is especially true for pyramided insecticidal traits. The phenotypic screen can identify individuals from the field that are homozygous for recessive resistance alleles before they become common, and it is possible to design specific resistance management measures to effectively reduce the impact such resistance may have. Dominant or codominant resistance alleles, which can increase at a faster rate than reces-

Table 2. Susceptibility (mortality and growth inhibition beyond first instar) of *H. virescens* neonates exposed to the Cry1F protein from *B. thuringiensis*

Colony	Slope ± SE	Significance of slope		Lc_{50} (ng/cm^2)		Goodnes of fit		Resistance ratio[a]	
		χ^2	Probability	Dose	95% FL	χ^2	Probability	RR_{50}	95% CL
GA1	0.36 ± 0.03	90.7	<0.0001	4.799	3.54–6.33	9.71	0.08	1.07	0.84–1.37
GA2	0.44 ± 0.03	185.4	<0.0001	3.320	2.86–3.81	3.17	0.67	0.74*	0.62–0.89
GA3	0.38 ± 0.02	185.2	<0.0001	5.073	4.36–5.86	2.15	0.82	1.14	0.94–1.36
LA1	0.33 ± 0.03	59.9	<0.0001	2.765	1.97–3.63	4.27	0.51	0.62*	0.45–0.85
LA2	0.46 ± 0.03	158.5	<0.0001	4.752	4.08–5.49	5.81	0.32	1.06	0.88–1.28
LA3	0.39 ± 0.03	130.1	<0.0001	2.860	2.33–3.38	7.12	0.21	0.64*	0.52–0.79
MS1	1.03 ± 0.11	79.9	<0.0001	4.632	3.28–5.96	9.48	0.09	1.48*	1.19–1.84
MS2	0.42 ± 0.03	112.3	<0.0001	4.931	4.09–5.88	3.91	0.56	1.10*	0.89–1.36
MS3	0.36 ± 0.03	141.9	<0.0001	6.487	5.47–7.67	6.27	0.28	1.45*	1.19–1.78
NC1	1.07 ± 0.16	40.2	<0.0001	6.352	4.06–8.70	13.31	0.02	2.01*	1.53–2.62
NC2	0.87 ± 0.13	43.8	<0.0001	7.473	5.55–9.41	9.08	0.10	1.67*	1.35–2.08
NC3	0.36 ± 0.04	68.4	<0.0001	3.712	2.81–4.73	1.60	0.90	0.83	0.63–1.10
TX1	0.41 ± 0.03	111.9	<0.0001	4.477	3.69–5.35	12.52	0.02	1.00	0.81–1.24
TX2	0.32 ± 0.03	98.6	<0.0001	8.233	6.64–10.20	6.91	0.22	1.85*	1.45–2.35
TX3	0.40 ± 0.03	105.2	<0.0001	7.970	6.64–9.57	3.60	0.60	1.79*	1.45–2.21
USDA–ARS	0.42 ± 0.02	348.7	<0.0001	4.448	4.00–4.92	8.67	0.12		

[a] Calculated by the formula of Robertson and Preisler (1992).
*Significantly different ($P < 0.05$) from the USDA–ARS reference colony.

sive alleles, can be identified at similarly low frequencies by both the F_2 screen and the phenotypic screen, again allowing an effective specific resistance management program to be implemented. The phenotypic screen based on a high diagnostic concentration is therefore sufficiently effective to adapt to reasonable susceptibility shifts allowing for resistance management actions to be designed and implemented to reduce the impact of Bt resistance.

Use of diet-overlaid rather than diet-incorporated insecticidal protein to generate baseline data enables long-term monitoring to be conducted at reasonable costs. Protein production and purification are very expensive, and quantities are limited. Diet overlay typically uses 2 orders of magnitude less protein than diet incorporation to produce repeatable results. Susceptibility baselines using diet overlay of protein have been established for other resistance monitoring programs for *B. thuringiensis* proteins in Lepidoptera, including heliothines (Marçon et al. 2000, Siegfried et al. 2000, Saeglitz et al. 2006, Sivasupramaniam et al. 2007, Bird and Ackhurst 2007). Consistency of methodology is essential in producing data that can be compared across time (Bird and Ackhurst 2007), so it is important that baseline studies such as this are conducted with consideration for the needs of the long-term monitoring program. Because changes in toxin batches might obscure changes in susceptibility it is important that baseline and monitoring methods use the same toxin batches as far as possible (Saeglitz et al. 2006). The speed, simplicity, and effectiveness of the phenotypic screen based on a high diagnostic concentration of diet-overlaid insecticidal protein makes it well suited for routine monitoring for field-relevant resistance in pest populations against which a Bt crop is highly effective.

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